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Inhibition of APC^{Cdh1} Activity by Cdh1/Acm1/Bmh1 Ternary Complex Formation*

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The anaphase-promoting complex (APC) is an essential E3 ubiquitin ligase responsible for catalyzing proteolysis of key regulatory proteins in the cell cycle. Cdh1 is a co-activator of the APC aiding in the onset and maintenance of G_1 phase, whereas phosphorylation of Cdh1 at the end of G_1 phase by cyclin-dependent kinases assists in the inactivation of APC^{Cdh1}. Here, we suggest additional components are involved in the inactivation of APC^{Cdh1} independent of Cdh1 phosphorylation. We have identified proteins known as Acm1 and Bmh1, which bind and form a ternary complex with Cdh1. The presence of phosphorylated Acm1 is critical for the ternary complex formation, and Acm1 is predominantly expressed in S phase when APC^{Cdh1} is inactive. The assembly of the ternary complex inhibits ubiquitination of Clb2 *in vitro* by blocking the interaction of Cdh1 with Clb2. *In vivo*, lethality caused by overexpression of constitutively active Cdh1 is rescued by overexpression of Acm1. Partially phosphorylated Cdh1 in the absence of *ACM1* still binds to and activates the APC. However, the addition of Acm1 decreases Clb2 ubiquitination when using either phosphorylated or non-phosphorylated Cdh1. Taken together, our results suggest an additional inactivation mechanism exists for APC^{Cdh1} that is independent of Cdh1 phosphorylation.

The eukaryotic cell cycle is largely controlled by targeted proteolysis of regulatory proteins, specifically through ubiquitin-mediated proteolysis (1–4). The anaphase-promoting complex (APC)[2] is an essential E3 ubiquitin ligase responsible for catalyzing proteolysis of key regulatory proteins in the cell cycle. Substrate specificity of the APC is attributed to co-activator proteins that associate with particular substrates throughout the cell cycle (5–7). The co-activators Cdc20 and Cdh1 are thought to aid in the recruitment of particular substrates through substrate binding independent of the APC or in assemblies known as APC^{Cdc20} or APC^{Cdh1} (8–10). The APC polyubiquitinates substrates, marking them for degradation by the proteasome (6, 11). Ubiquitin ligase activity of the APC has been shown to require the presence of Cdc20 or Cdh1 (5, 11–14). Co-activators interact with the APC at specific times during the cell cycle, with Cdc20 activating the APC from early to late M phase and Cdh1 activating the APC from late M phase through G_1 (5, 12, 15).

Phosphorylation of Cdh1 has been demonstrated as an important control mechanism for the APC^{Cdh1} complex (16). Phosphorylation of Cdh1 is cell cycle-dependent and is considered to be the main regulatory mechanism by which APC^{Cdh1} is inactivated. Cdh1 is phosphorylated in S phase, G_2, and mitosis and dephosphorylated in G_1 (16). The phosphorylation of Cdh1 corresponds to high levels of the cyclin-dependent kinase (CDK) activity. When the CDK consensus phosphorylation sites within Cdh1 from budding yeast were mutated to alanine, Cdh1 activated the APC constitutively, failing to accumulate mitotic cyclins (16). Cdh1 is phosphorylated at the G_1/S phase transition by G_1 and S phase cyclins that activate the CDK in budding yeast, Cdc28. Cdh1 remains hyperphosphorylated until late in mitosis when Cdc14 phosphatase is activated allowing Cdh1 to reactivate the APC (15, 17). In S and M phases, Cdh1 failed to associate with the APC in Cdh1 co-immunoprecipitations (16). Additionally, *in vitro* phosphorylation of recombinant Cdh1 by CDKs is sufficient for inactivation of the APC (18).

Protein-protein interactions have also been shown to be important in the regulation of Cdh1 (19, 20). In higher eukaryotes, Emi1 and Mad2B have been shown to inhibit Cdh1 through protein-protein interactions (19, 20). Although the function of Mad2B is unclear, Emi1 is reported to inhibit substrate binding to Cdh1. However, similar inhibitors in yeast have not yet been identified.

To discover potentially novel Cdh1 interacting proteins, we performed a proteomic screen of Cdh1 immunoprecipitations in budding yeast. In the process, we identified a multiprotein complex that forms with Cdh1, consisting of a previously uncharacterized protein Acm1 (YPL267W) and two members of the 14-3-3 protein family known as Bmh1 and Bmh2. The expression of Acm1 is cell cycle-dependent, and Acm1 is necessary for complex formation with Cdh1 and Bmh1. Formation of the complex, which we refer to as the CAB complex or Cdh1/Acm1/Bmh1 complex, is restricted to the cell cycle window in which Acm1 is expressed. Without Acm1, Clb2 levels *in vivo* appear lower when compared with cells containing Acm1 under certain growth conditions. Complex formation inhibits

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[2] The abbreviations used are: APC, anaphase-promoting complex; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; CDK, cyclin-dependent kinase; CAB complex, complex of Cdh1 Acm1 and Bmh1; GST, glutathione *S*-transferase; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; TOF, time-of-flight; WT, wild type; FACS, fluorescence-activated cell sorting.

TABLE 1
***S. cerevisiae* strains used in this study**

Strain	Relevant genotype	Source
W1588-4c	*MAT*a *ade2-1 can1–100 His3–11 15 leu2–3 112 trp1-1 ura3-1*	R. Rothstein
YKA150	W1588-4c *bar1*Δ::*URA3*	Hall *et al.* (23)
YKA154	YKA150 *CDH1-3FLAG*::KanMX4	This study
YKA323	YKA150 *ypl267w*Δ::KanMX4	This study
DLY3033	*MAT*a *bar1*::*URA3 cdc15-2 ura3 leu2 trp1*	J. Pringle
YKA156	DLY3033 *CDC27-3FLAG*::KanMX4	This study
BY4741	*MAT*a *his3-D1 leu2-D0 met15-D0 ura3-D0*	Open Biosystems
YPL267W-TAP	BY4741 *Acm1-TAP*::HIS3MX6	Open Biosystems
YKA322	YPL267W-TAP *cdh1*Δ::KanMX4	This study
YER177W-TAP	BY4741 *Bmh1 -TAP*::HIS3MX6	Open Biosystems
YNL172W-TAP	BY4741 *APC1-TAP*::HIS3MX6	Open Biosystems
YGL003CΔ	BY4741 *cdh1*Δ::KanMX4	Open Biosystems
YPL267WΔ	BY4741 *acm1*Δ::KanMX4	Open Biosystems
YER177WΔ	BY4741 *bmh1*Δ::KanMX4	Open Biosystems
YDR099WΔ	BY4741 *bmh2*Δ::KanMX4	Open Biosystems

APC^{Cdh1} activity *in vitro*, and lethality caused by overexpression of constitutively active Cdh1 is rescued by Acm1 overexpression. The CAB complex functions to block substrate binding with the co-activator. Partially phosphorylated Cdh1 binds and activates the APC in the absence of Acm1. Lastly, we have shown the ability of Acm1 and Bmh1 to inhibit APC^{Cdh1} despite the Cdh1 phosphorylation state. Taken together, our data suggest the existence of an additional inactivation mechanism of APC^{Cdh1} independent of Cdh1 phosphorylation.

EXPERIMENTAL PROCEDURES

Cloning—Cloning for p415ADH-FLAGCdh1 and pNC219-FLAGCdh1m9 have been described previously (21). The resulting centromeric plasmid, p415ADH-FLAGCdh1, constitutively expresses 3×FLAG-*CDH1* from the *ADH* promoter. pNC219-FLAGCdh1m9 expresses the 3×FLAG-*CDH1* phosphomutant with 9 of 11 CDK sites mutated to alanine from the *GAL* promoter. The plasmids pHLP117 expressing 3HA-Acm1 from its natural promoter, pHLP107 expressing FLAG-Acm1 from the *ADH* promoter, and pHLP106 expressing FLAG-Acm1 from the *GAL1* promoter were gifts from M. Hall. The plasmids pRSETClb2, pET28-His_6-Cdh1, and pET28-His_6-Ubc4 were gifts from D. Barford (22). *BMH1* and *CLB2* were amplified by PCR from yeast genomic DNA and cloned into pGEX-4T-1 at the BamH1 and XhoI sites yielding a GST tag on the N terminus of Bmh1 and Clb2 for overexpression in *Escherichia coli*. All PCR-generated constructs were confirmed by DNA sequencing.

Strains and Media—Yeast strains are described in Table 1. Media were YPD (20 g/liter peptone (Fisher), 10 g/liter yeast extract (Bacto), 20 g/liter dextrose (MP Biomedicals)), YP (20 g/liter peptone, 10 g/liter yeast extract) and synthetic medium (6.7 g/liter yeast nitrogen base lacking amino acids (Difco), 20 g/liter dextrose, or 20 g/liter raffinose, appropriate amino acid dropout mix (Q-Biogene)).

Cell Cycle Arrest—For G_1 arrest in *bar1* cells, α-factor peptide is added directly to the cultures during mid-log phase growth at 50 μg/liter. *BAR1* cells require 3 μg/ml α-factor peptide for an effective G_1 arrest. Arresting in S phase requires the addition of 10 mg/ml hydroxyurea (Sigma). M phase arrests are carried out by the addition of 15 mg/ml nocodazole (Sigma). This laboratory has acquired a *cdc15-2* yeast strain that allows cell cycle arrest in telophase induced by temperature shift from 25 °C to 37 °C. Cell cycle arrests are monitored by phase-contrast microscopy until >90% of the cells have achieved the desired morphology.

Cdh1 Purification—Yeast cells were grown asynchronously to A_{600} = 1 and lysed in 1 volume of APC-C lysis buffer (25 mM HEPES-NaOH, pH 7.5, 400 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture (Roche Applied Science)) by vortexing for 45 min at 4 °C in 1.7-ml microcentrifuge tubes containing 0.5 ml of 0.5-mm glass beads. Cell lysate was cleared by centrifugation at 16,000 × *g* for 15 min. Soluble extracts were pooled and cleared a second time at 5000 rpm for 5 min. Extracts were incubated with anti-FLAG M2 antibody-coupled resin (Sigma) for 1.5 h at 4 °C. Bound Cdh1 was washed extensively with APC-C buffer (25 mM HEPES-NaOH, pH 7.5, 400 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and eluted 2× with FLAG peptide (250 μg/ml) in APC-C buffer.

APC Purification—Yeast cells were grown and lysed, and lysates were cleared in the same manner as for Cdh1. Extracts from YNL172W-TAP were incubated with calmodulin affinity resin (Stratagene) for 1.5 h at 4 °C in APC-C buffer containing 1 mM $CaCl_2$. Cells from YKA156 (2 liters) were grown to A_{600} = 0.5 at 25 °C, and temperature was shifted to 37 °C for 2 h. Extracts from YKA156 were incubated with EZView anti-FLAG M2 antibody-coupled resin (Sigma) for 1.5 h at 4 °C, washed extensively with APC-C buffer, and eluted 2× with FLAG peptide (250 μg/ml) in low salt APC-C buffer (25 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.1 mM dithiothreitol).

Acm1 Purification—Yeast cells were grown and lysed, and lysates were cleared in the same manner as for Cdh1 purifications. Extracts from YPL267W-TAP were incubated with calmodulin affinity resin (Stratagene) for 1.5 h at 4 °C in APC-C buffer containing 1 mM $CaCl_2$. Acm1 bound to beads was used in binding assays. Acm1 used in the ubiquitination assay and Clb2 binding assay was eluted with TAP elution buffer (40 mM Tris, pH 7.5, 0.6 mM dithiothreitol, 2.5 mM EDTA) for 30 min at room temperature while shaking at 700 rpm. $MgCl_2$ was added to the elution buffer at a final concentration of 15 mM. FLAG-

Acm1 used in the ubiquitination assay and binding assays was purified in the same manner as APC.

Bmh1/Clb2 Purification—Overnight cultures of *E. coli* harboring *BMH1* or *CLB2* in the pGEX-4T-1 plasmid were grown and used to inoculate 1 liter of 2XYT (16 g/liter Tryptone, 10 g/liter yeast extract, 5 g/liter NaCl) containing 100 μg/ml ampicillin. Cultures were grown for 8 h at 37 °C, temperature was shifted to 23 °C, and isopropyl 1-thio-β-D-galactopyranoside was added (100 μM final contration). Cells were grown overnight and harvested by centrifugation. The resulting cell pellet was resuspended in 1× phosphate-buffered saline 0.5 M NaCl keeping suspension on ice. Cells were lysed 3× using sonication for 20-s intervals with a 40-s pause. Lysates were cleared by centrifugation at 4 °C and 25,000 rpm for 30 min. Cleared lysates were incubated with 100 μl of washed GSH beads (glutathione-Sepharose 4B, Amersham Biosciences) for 10 min. Beads and lysate were pipetted and transferred to Bio-Rad micro columns. Beads were washed with 5 column volumes of 1× phosphate-buffered saline 0.5 M NaCl. Bound protein was used on the GSH beads for binding assays. Bmh1 used in the ubiquitination assay was treated with biotinylated thrombin (Novagen) to cleave the GST tag. The sample was treated with streptavidin-agarose to remove thrombin. Buffer was exchanged with 40 mM Tris, pH 7.5, 0.6 mM dithiothreitol, 10 mM $MgCl_2$ using Microcon filters with a 10-kDa cutoff (Millipore).

In Vitro Binding Assays—Compact reaction columns (USB) were used for all binding assays. Protein used as bait was immobilized on affinity beads and washed in the reaction column. Protein used as prey was added to each column and allowed to incubate for times noted at 4 °C under rotation. Columns were washed with five column volumes of APC-C lysis buffer and eluted in 1× NuPAGE LDS sample buffer (Invitrogen) unless the immobilized protein used the TAP tag. In TAP purifications, APC-C buffer containing 1 mM $CaCl_2$ was used for washes. Five percent of each reaction was loaded on the gel.

Ubiquitination Assay—Ubiquitination assays were carried out as described by D. Barford (22). The substrate Clb2 (pRSETClb2), the co-activator Cdh1 (pET28-His_6-Cdh1), and the E2 Ubc4 (pET28-His_6-Ubc4) were prepared using the TNT T7 Quick-coupled *in vitro* transcription/translation kit (Promega) unless otherwise noted. APC (YKA156) and Acm1 were purified from budding yeast (see APC and Acm1 purification), whereas Bmh1 was expressed and purified from *E. coli* (see Bmh1 purification). APC used in each reaction was 1/50th of the elution from 2 liter of cells. Clb2 was radiolabeled with [^{35}S]methionine (PerkinElmer Life Sciences). Reactions were run on a 4–12% Bis-Tris NuPAGE gel (Invitrogen), dried, and imaged using Hyperfilm (Amersham Biosciences).

Flow Cytometry—Cells were prepared as described previously (23). DNA content was measured on a FACScan instrument and ModFit LT software (Verity Software House, Inc.) used to calculate percentages of G_1, S, and G_2/M cells present.

Protein Identification and Mass Spectrometry—Elutions from Cdh1 purifications were acetone-precipitated, reconstituted in 1× LDS sample buffer containing 100 mM dithiothreitol, and separated on a 4–12% Bis-Tris NuPAGE gel (Invitrogen). Gels were stained with Coomassie Blue or silver. Protein bands were excised, destained, and incubated with trypsin. After digestion, samples were frozen, lyophilized, and resuspended in a small volume of 50% methanol/0.1% formic acid. Peptide masses were analyzed on a matrix-assisted laser desorption ionization-TOF/TOF mass spectrometer (Applied Biosystems), and proteins were identified using the MASCOT search engine (Matrix Science). Samples that needed further analysis were analyzed on a nano-ESI Q-TOF (Micromass) mass spectrometer (47).

Gel Filtration—FLAG immunoprecipitations were prepared from S phase-arrested cells in APC-C buffer. The samples were fractionated on a Superdex 200 HR 10/30 gel-filtration column with extraction buffer on an AKTA Explorer system (Amersham Biosciences) at 0.4 ml/min. Fractions were collected in 0.5-ml increments, and each fraction was acetone-precipitated. Proteins were resolved by SDS-PAGE and detected by immunoblotting with an α-FLAG antibody.

RESULTS

Acm1 and Bmh1 Associate with Cdh1 in Vivo and in Vitro and Form a Ternary Complex—To identify potential interacting proteins of Cdh1, we used a targeted proteomic approach consisting of immunoprecipitations with genomically FLAG-tagged Cdh1 followed by mass spectrometric analysis. We discovered three proteins known as Acm1, Bmh1, and Bmh2 in budding yeast, which stably interact with Cdh1 under high salt wash conditions (Fig. 1*A*). The untagged parent strain was used as a control. C-terminal Cdh1 fragments were also identified by mass spectrometry. Interestingly, we did not identify any other specific proteins in the Cdh1 immunoprecipitation presumably due to the high salt wash conditions, which disrupt all but the most stable protein complexes. Gel-filtration analysis of the complex showed co-elution of Cdh1 and Acm1 in a stoichiometric interaction (data not shown). Acm1 (APC^{Cdh1} modulator 1) is a previously uncharacterized protein with no known function, although it was originally identified as a potential CDK substrate (24). However, as members of the 14-3-3 family of proteins, Bmh1 and Bmh2 have been associated with a variety of cellular processes and have been defined as phosphoserine-binding proteins (25, 26). We believe two possibilities for these interactions exist, either as potential APC^{Cdh1} substrates or as regulators of Cdh1.

To confirm the specificity of the interactions between Cdh1, Acm1, and Bmh1, we used *in vitro* binding assays. FLAG-tagged Cdh1 and TAP-tagged Acm1 were purified from yeast while recombinant Bmh1 was expressed in *E. coli*. GST-Bmh1 was immobilized on GSH beads and incubated with Cdh1-FLAG. GST bound to GSH beads was used as a control. Acm1-TAP was immobilized on calmodulin beads and incubated with Cdh1-FLAG. Empty calmodulin beads served as a control. Likewise, Acm1-TAP was incubated with GST-Bmh1. We observed stable interactions between Bmh1 and Cdh1, Acm1 and Cdh1, and Acm1 and Bmh1 under high salt wash conditions (Fig. 1*B*). To see if any one component of the CAB complex was necessary for complex formation, we performed immunoprecipitations from strains deleted for components of the complex. We expressed N-terminally FLAG-tagged Cdh1 in WT, *acm1*Δ, *bmh1*Δ, and *bmh2*Δ strains and immunopurified it with anti-FLAG antibody followed by gel electrophoresis and mass spec-

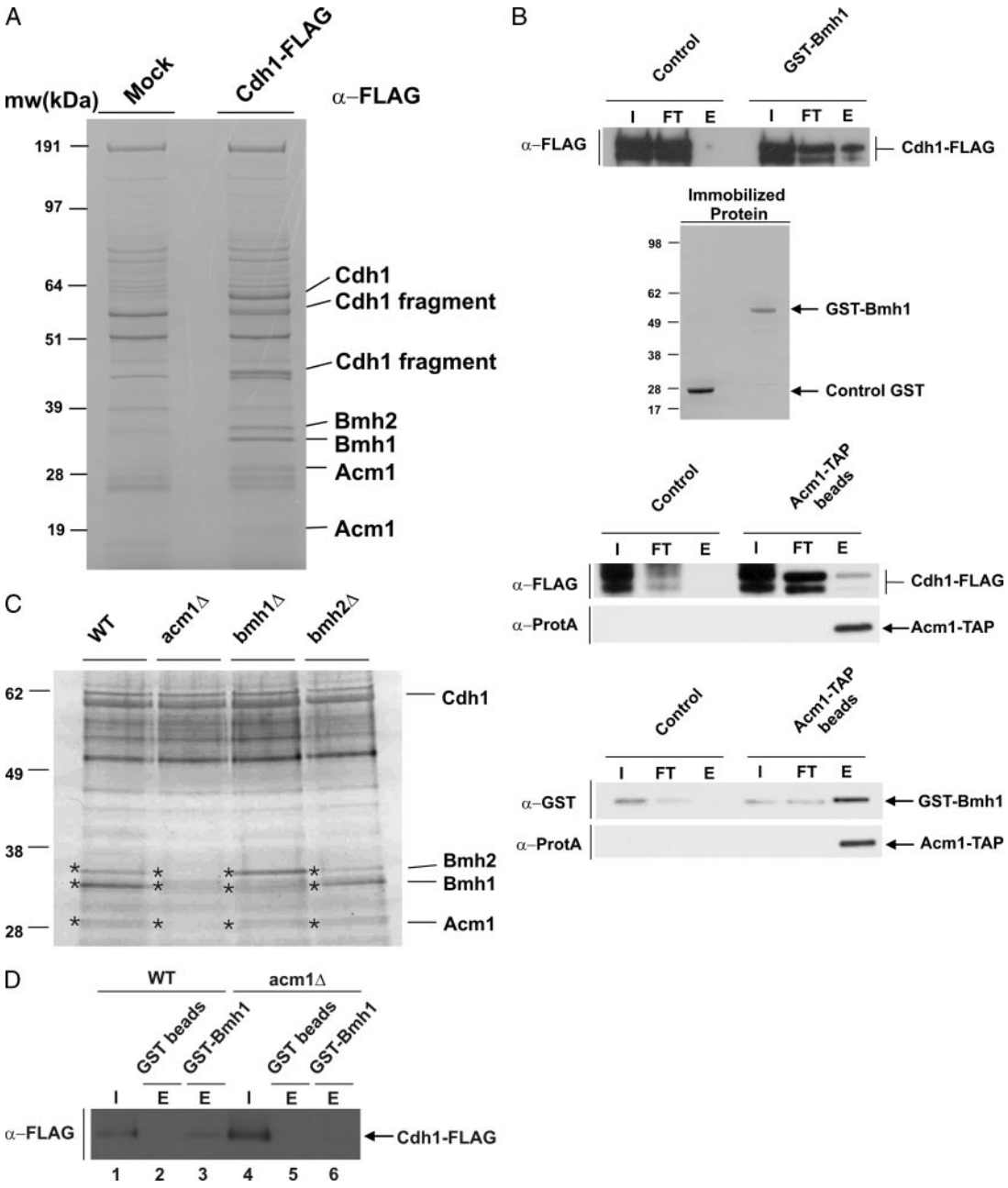


FIGURE 1. **Acm1, Bmh1, and Cdh1 form a ternary complex.** *A*, immunoprecipitations were performed with the yeast strain YKA154 containing Cdh1 with a 3×FLAG epitope at the C terminus. The untagged parent strain was used as a control. Four liters of cells were grown to mid-log phase and arrested in G_1 with α-factor. Once harvested, cells were lysed and subjected to antibody affinity pull-down assays using 100 μl of the α-FLAG antibody beads. Samples were run on 4–12% Bis-Tris gels (Invitrogen) and Coomassie -stained. From each lane, gel slices were excised throughout the entire length of the gel. Proteins in each gel slice were digested with trypsin, analyzed by mass spectrometry, and identified through subsequent data base searching. Those proteins present in the sample lane and absent in the control lane were considered to interact with Cdh1. Three proteins known as Bmh1, Bmh2, and a previously uncharacterized protein, Acm1, were identified as Cdh1-interacting proteins. *B*, GST-Bmh1 from *E. coli* immobilized on 10 μl of GSH beads or Acm1-TAP from 1 liter of asynchronous yeast cells (A_{600} = 1) immobilized on 60 μl of calmodulin affinity beads were used in the compact reaction columns for the respective binding assay. 10 μl of purified Cdh1-FLAG from 1 liter of asynchronous yeast cells or 10 μl of purified GST-Bmh1 were incubated with the immobilized protein for 2 h at 4 °C and washed extensively with high salt buffer. Bound proteins were eluted in 1× LDS buffer, run on 4–12% Bis-Tris gels, and transferred to membranes for Western analysis. *I*, *FT*, and *E* designate input, flow-through, and elution. GST immobilized on GSH beads or empty calmodulin beads were used as controls. The immobilized protein shown is Coomassie-stained and is 20× greater than that shown in binding assay. Cdh1 bound to Bmh1 and Acm1, and Bmh1 bound to Acm1. *C*, Cdh1, harboring an N-terminal 3×FLAG epitope transcribed from the *ADH* promoter on a centromeric plasmid, was expressed by transforming the plasmid into WT, *acm1*Δ, *bmh1*Δ, and *bmh2*Δ strains. Each strain was grown asynchronously in 1 liter of selective media to A_{600} = 1 before harvesting and pulling down Cdh1 with 25 μl of anti-FLAG M2 antibody-coupled resin (Sigma). Proteins were separated on a 4–12% Bis-Tris gel and Coomassie-stained. *, gel slices from each lane were excised in the region for which each protein migrates, digested with trypsin, and analyzed by mass spectrometry to detect the presence of peptides corresponding to the proteins of interest. Neither Bmh1 nor Bmh2 could be detected in the *acm1*Δ strain. Bmh1 peptides could not be detected in *bmh1*Δ, but Acm1 and Bmh2 were present. Likewise, Bmh2 could not be detected in *bmh2*Δ, but Acm1 and Bmh1 were present. *D*, Cdh1 was purified from 2 liter of asynchronous cells grown to A_{600} = 1 from either WT or *acm1*Δ strains using 50 μl of α-FLAG antibody beads. GST-Bmh1 was immobilized on 5 μl of GSH beads in compact reaction columns. 2 μl of FLAG-Cdh1 purified from WT and *acm1*Δ cells was incubated with the immobilized protein for 2 h at 4 °C and washed extensively with high salt buffer. Bound proteins are eluted in 1× LDS buffer, run on 4–12% Bis-Tris gels, and transferred to membranes for Western analysis. An α-FLAG antibody was used to detect the presence of Cdh1. The absence of Acm1 abolished the Bmh1-Cdh1 interaction.

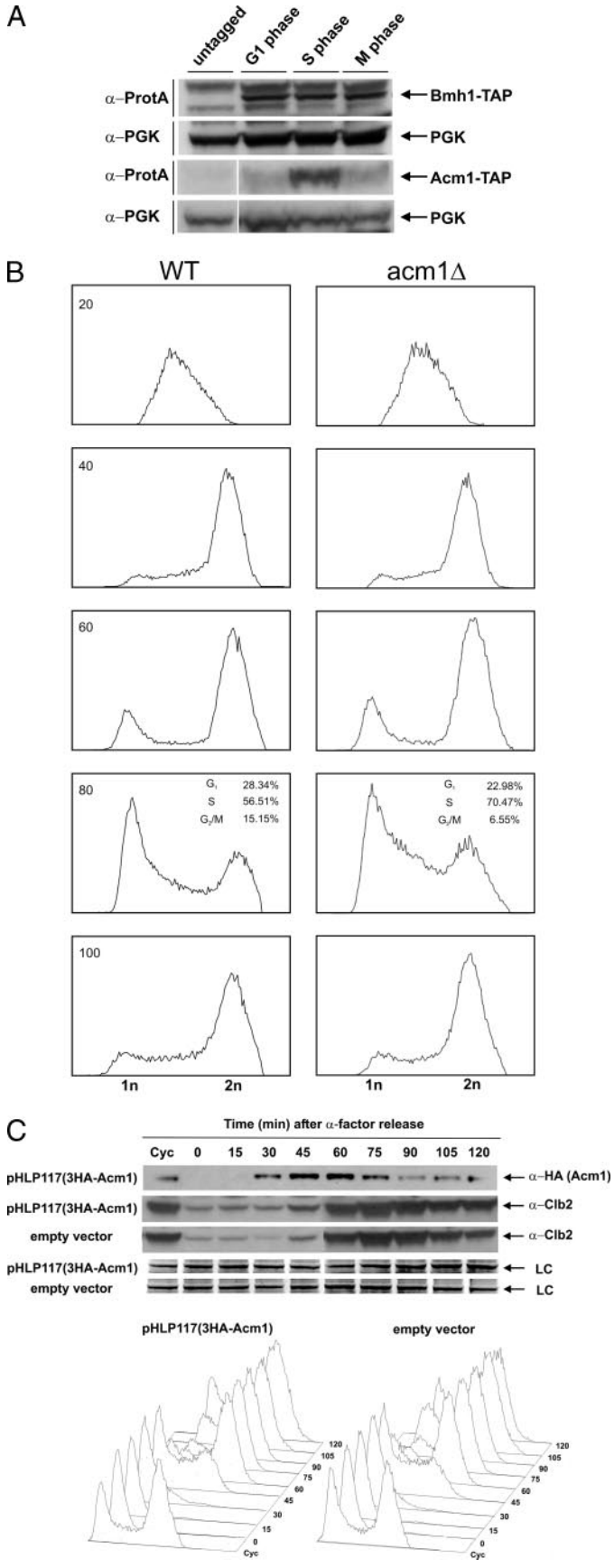
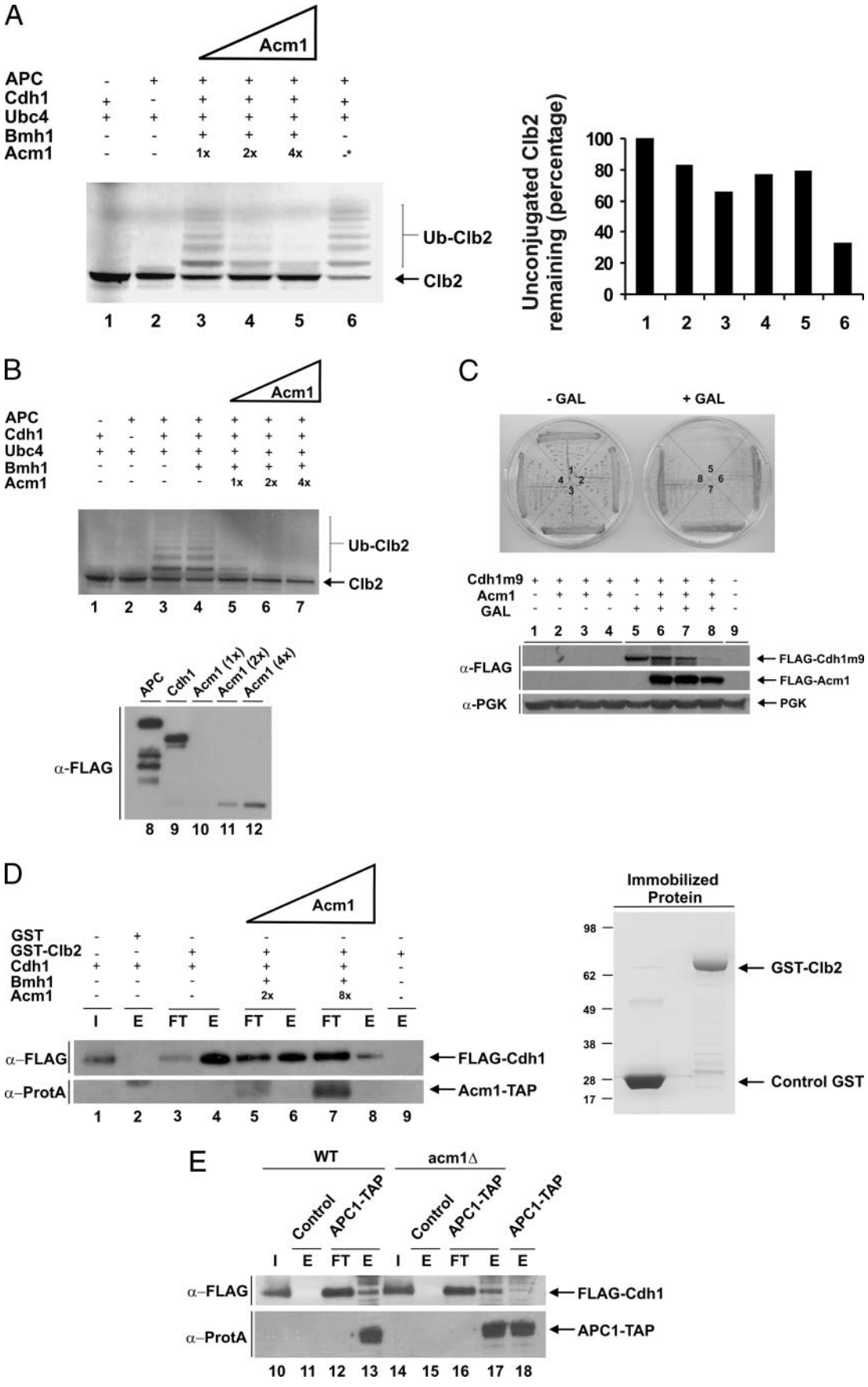


FIGURE 2. **Acm1 is expressed in S phase, and the deletion of Acm1 leads to an enriched S-phase cell population.** *A*, strains containing either Acm1 or Bmh1 with a C-terminal TAP genomic tag were grown to mid-log phase and arrested in G_1 with α-factor, S phase with hydroxyurea, and M phase with nocodazole. Cells were harvested and lysed, and extracts were run on 4–12% Bis-Tris gels. Western analysis using an anti-Protein A antibody was performed to measure protein levels in each cell cycle phase. Acm1 levels are highest in S phase, whereas Bmh1 levels remain constant throughout the cell cycle. *B*, cells from WT and *acm1*Δ strains were arrested with hydroxyurea for synchronization and released into fresh media. Samples were collected in 20-min intervals for 100 min after release. DNA was stained using SYTOX Green (Molecular Probes), and DNA content was measured on a FACScan instrument for cell cycle progression. Time points for the FACS analysis are present from 20 to 100 min. An enriched S phase population was observed at 80 min in the *acm1*Δ strain when compared with WT cells. Percentages of G_1, S, and G_2/M cells for WT and *acm1*Δ strains at 80 min are shown. ModFit LT software (Verity Software House, Inc.) was used to calculate percentages of G_1, S, and G_2/M cells. *C*, cells from the *acm1*Δ strain (YKA323) containing the plasmid pHLP117 expressing HA-Acm1 from its natural promoter or cells from the same strain containing the empty plasmid were grown to mid-log phase and arrested in G_1 with α-factor. Cells were released from the G_1 arrest by washing with 4× the culture volume (fresh media) and resuspending washed cells in fresh media. Time points were taken in 15-min intervals. 40 μg of extract was loaded on the gels and probed with an α-HA antibody or an α-Clb2 antibody. The Coomassie-stained membranes were used as loading controls. A decrease in Clb2 levels is seen from time points 0–45 min in cells lacking Acm1. FACS analysis was performed for each 15-min time point of the G_1 arrest and release.

trometric analysis. Gel slices for each region where Cdh1, Acm1, Bmh1, and Bmh2 migrate were analyzed. Bmh1 and Bmh2 cannot be detected in the absence of Acm1. In the *bmh1*Δ strain, Acm1 and Bmh2 still associated with Cdh1. Likewise, Acm1 and Bmh1 still associated with Cdh1 in the *bmh2*Δ strain (Fig. 1*C*). Additionally, we incubated purified FLAG-Cdh1 from either wild-type or *acm1*Δ cells with immobilized GST-Bmh1 in an *in vitro* binding assay. Without Acm1, Cdh1 does not bind Bmh1 (Fig. 1*D*, *lane 6*). The Bmh1/Cdh1 binding in Fig. 1*B* is attributed to the presence of Acm1, which co-purifies with Cdh1. Together these findings illustrate the dependence of the CAB complex formation on Acm1 as well as a possible redundancy between Bmh1 and Bmh2.

Acm1 Is Expressed in S Phase and Has an Effect on Cell Cycle Progression—Because Cdh1 activates the APC in a cell cycle-dependent manner, the interaction with Acm1 and the 14-3-3 proteins could also fluctuate throughout the cell cycle. To determine whether these proteins were regulated in a cell cycle-dependent manner, Acm1-TAP and Bmh1-TAP protein levels were analyzed in cell lysates from G_1, S, and M phase-arrested cells. Acm1 levels were found to be significantly higher in S phase over the other cell cycle phases, whereas Bmh1 levels remain constant in each cell cycle stage (Fig. 2*A*). This finding is in agreement with the Acm1 mRNA expression pattern throughout the cell cycle (27). Analysis of Acm1 mRNA expression levels from the *Saccharomyces* Genome Database revealed the closest pattern of gene expression to Acm1 is observed for Clb5 (27). Clb5-Cdc28 is believed to be the primary cyclin-CDK complex responsible for phosphorylation of Cdh1 during S phase, although other G_1 cyclin-CDK complexes are also involved (15, 28, 29). Acm1 protein levels are highest in S phase when Cdh1 is phosphorylated and APC^{Cdh1} becomes inactivated. This observation suggested an involvement of Acm1 in phosphorylation-mediated regulation of APC^{Cdh1} during S phase.

We did not expect the deletion of *ACM1* to induce large perturbations in the cell cycle. According to the *Saccharomyces* Genome Database, cells with *ACM1* systematically deleted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Acm1
APC
Cdh1
Ubc4
Bmh1
Acm1
1x
2x
4x
Ub-Clb2
Clb2
1 2 3 4 5 6
Unconjugated Clb2 remaining (percentage)
100
80
60
40
20
0
1 2 3 4 5 6
B
Acm1
APC
Cdh1
Ubc4
Bmh1
Acm1
1x
2x
4x
Ub-Clb2
Clb2
1 2 3 4 5 6 7
APC
Cdh1
Acm1 (1x)
Acm1 (2x)
Acm1 (4x)
α-FLAG
8 9 10 11 12
C
- GAL
+ GAL
Cdh1m9
Acm1
GAL
1 2 3 4 5 6 7 8 9
α-FLAG
FLAG-Cdh1m9
FLAG-Acm1
α-PGK
PGK
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Acm1
GST
GST-Clb2
Cdh1
Bmh1
Acm1
2x
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I E FT E FT E FT E E
α–FLAG
FLAG-Cdh1
α–ProtA
Acm1-TAP
1 2 3 4 5 6 7 8 9
Immobilized Protein
98
62
49
38
28
17
GST-Clb2
Control GST
E
WT
acm1Δ
Control
APC1-TAP
Control
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APC1-TAP
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α–FLAG
FLAG-Cdh1
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APC1-TAP
10 11 12 13 14 15 16 17 18

were viable (30). Indeed, *acm1*Δ cells grow at normal rate when compared with WT cells. To see if deleting *ACM1* had any effect within a single cell cycle, cells from *acm1*Δ and wild-type strains were synchronized in S phase with hydroxyurea, released, and analyzed by flow cytometry for DNA content in 20-min intervals (Fig. 2*B*). Interestingly, FACS analysis of *acm1*Δ cells showed an increase (14%) in the S phase cell population in comparison to wild-type cells at 80 min after release. This effect was reproducible but to slightly varying degrees (data not shown). Additionally, the *acm1*Δ strain resynchronized with WT cells in M phase. The effect in S phase progression in *acm1*Δ cells suggests Acm1 may play a role in cell cycle progression throughout S phase. If the CAB complex does play a role in APC^{Cdh1} regulation, one would expect a difference in APC^{Cdh1} substrate levels in cells lacking Acm1. To test whether APC^{Cdh1} substrates are affected in *acm1*Δ cells, we performed a G_1 block-release experiment in cells containing Acm1 compared with cells void of Acm1 and examined levels of the known APC^{Cdh1} substrate Clb2. Interestingly, we found a slight decrease in Clb2 levels in early time points in the absence of Acm1 under certain growth conditions (Fig. 2*C*). The cycling pattern of Clb2 corresponds to that of Acm1, although Acm1 increases to its maximum slightly before Clb2 reaches its highest level. An increased level of APC^{Cdh1} activity in the strain lacking Acm1 could explain this difference in Clb2 levels between the two strains. However, the effects in the cell cycle are slight, so CAB complex formation may exist as an overlapping inhibitory mechanism in addition to phosphorylation for APC^{Cdh1}.

CAB Complex Formation Inhibits APC^{Cdh1}-mediated Ubiquitination of Clb2—Because Acm1 was predominantly expressed in S phase when APC^{Cdh1} activity is inhibited and cells lacking Acm1 exhibited a difference in Clb2 levels, we investigated whether the CAB complex formation affected APC^{Cdh1} activity directly. To demonstrate a possible inhibitory effect of Acm1 and Bmh1 on APC activity, an *in vitro* ubiquitination assay was used to measure ubiquitination of the substrate Clb2 (Fig. 3*A*). APC and TAP-tagged Acm1 were purified from yeast while Ubc4 (E2), Cdh1, and S^{35}-labeled Clb2 were expressed *in vitro* and incubated together for the assay. The degree of substrate ubiquitination was measured by phosphorimaging analysis of S^{35}-labeled Clb2. With Bmh1 levels held constant, increasing concentrations of Acm1-TAP resulted in a dose-dependent inhibition of Clb2 ubiquitination (*lanes 3–5*). A mock purification from *acm1*Δ cells was used to rule out buffer or nonspecific protein effects on APC^{Cdh1} activity and illustrates Clb2 ubiquitination levels without any inhibition (*lane 6*). To quantify Acm1 inhibition relative to APC and Cdh1 levels, we used yeast purified FLAG-tagged APC, Cdh1, and Acm1 (Fig. 3*B*). Acm1 appears to inhibit APC activity at substoichiometric levels (Fig. 3*B*, *lanes 5–7* and *10–12*), although we have observed stoichiometric binding from our gel-filtration data. We attribute this result to the presence of a certain percentage of inactive Cdh1 in the preparation. No inhibitory effect on Clb2 ubiquitination was observed upon the addition of Bmh1 alone (Fig. 3*B*, *lane 4*). This is not surprising due to the requirement of Acm1 for the Bmh1-Cdh1 interaction. From our *in vitro* ubiquitination assay results, we conclude Acm1 and Bmh1 have an inhibitory effect on APC^{Cdh1} ubiquitin ligase activity.

To investigate whether Acm1 inhibits APC^{Cdh1} activity *in vivo*, we co-expressed Acm1 and the constitutively active Cdh1 mutant containing 9 of the 11 CDK phosphorylation sites mutated to alanine. It has been previously shown that Cdh1 phosphomutants mimicking non-phosphorylated Cdh1 were unable to accumulate Clb2 and Clb3, to form mitotic spindles, and undergo cytokinesis (16). We found overexpression of Acm1 rescues the lethality caused by overexpression of the Cdh1 phosphomutant (Fig. 3*C*). From this experiment, we concluded the CAB complex formation suppresses APC^{Cdh1} activity as an overlapping regulatory mechanism in the absence of complete CDK phosphorylation. This mechanism exists in addition to Cdh1 phosphorylation, which has clearly been shown to also inhibit APC^{Cdh1} activity. CAB complex forma-

FIGURE 3. **Acm1/Bmh1 inhibit APC^{Cdh1} ubiquitin ligase activity by blocking Cdh1 binding to the APC.** *A*, Cdh1, Ubc4, and Clb2 used in this assay were produced in the TnT T7 Quick-coupled *in vitro* transcription/translation kit, and Clb2 was labeled with [^{35}S]methionine. APC was purified from yeast and Bmh1 from *E. coli*. Acm1 was purified from 2 liters of asynchronous cells grown to $A_{600} = 1$ using 100 μl of washed calmodulin resin. Resin was washed extensively with APC-C buffer containing 1 mM $CaCl_2$ and eluted in 2.5 mM EDTA buffer. 15 mM $MgCl_2$ was added to the eluted Acm1 before use in the ubiquitination assay. Components of the assay were mixed and incubated as described previously (22). Ubiquitination of Clb2 can be seen as bands of decreasing gel mobility. The *triangle* represents increasing levels of Acm1. 1× Acm1 is the equivalent of 2 μl or 1/50 from the EDTA elution. Acm1 inhibits APC^{Cdh1} activity in a dose-dependent manner. *, control purification from the *acm1*Δ strain equivalent to 4× concentration of Acm1 added. Levels of unconjugated Clb2 for the quantitation of APC^{Cdh1} inhibition by CAB complex formation are shown in the *right panel*. *B*, ubiquitination assay is the same as above with the exception of using Cdh1 (p415ADH-FLAGCdh1 in YPL267WΔ) and Acm1 (pHLP107 in YGL003Δ) purified from yeast using FLAG immunoprecipitations for quantitation. Ubc4 and Clb2 produced in the *in vitro* transcription/translation kit. Bmh1 levels used in the assay are sufficient to see by Coomassie staining. Levels of APC, Cdh1, and Acm1 used in each assay can be seen in the *lower panel*. *C*, Cdh1 containing 9 of 11 CDK sites mutated to alanine was overexpressed through use of the *GAL* promoter (pNC219-FLAGCdh1m9 in YKA323). In the same strain, pHLP106 expressing FLAG-Acm1 from the *GAL* promoter was also transformed. Cells were plated on SD-Trp and grown for 48 h. Colonies were selected and plated on Raf-Trp, grown for 48 h, and replica-plated to Raf-Trp media containing galactose. Extracts (40 μg) were separated by SDS-PAGE and immunoblotted with α-FLAG and PGK antibodies. Cdh1m9 overexpression is lethal when expressed without Acm1 (*section 5* and *lane 5*), but lethality is rescued by Acm1 overexpression (*sections 6* and *7*, *lanes 6* and *7*). Three colonies were tested to ensure each contained both transformed plasmids. *Sections 6–8* contain both plasmids, although *section 8* exhibited low Cdh1m9 expression. Extract from the control strain without plasmids present is shown in *lane 9*. *D*, bacterially expressed GST-Clb2 was incubated with FLAG-Cdh1 from *acm1*Δ cells for 30 min at 4 °C after Cdh1 was preincubated with varying levels of Acm1. Clb2 was immobilized on affinity beads. GST bound to GSH beads and GST-Clb2 without Cdh1 were used as controls. Following incubation with Cdh1, columns were washed extensively with high salt APC-C buffer and eluted with 1× LDS sample buffer. Samples were run on a 4–12% Bis-Tris gel and transferred for Western blot analysis. Immobilized protein shown is Coomassie-stained and is 20× greater than that shown in binding assay. *I*, *FT*, and *E* designate input, flow through, and elution. Antibodies used for detection are as noted. Clb2 binding to Cdh1 decreases with an increasing concentration of Acm1. *E*, N-terminally tagged FLAG-Cdh1 was purified from either WT or *acm1*Δ cells and incubated with APC via Apc1-TAP for 30 min at 4 °C. Empty calmodulin beads and APC-TAP without Cdh1 were used as controls. Following incubation with Cdh1, columns were washed extensively with high salt APC-C buffer and eluted with 1× LDS sample buffer. Samples were run on a 4–12% Bis-Tris gel and transferred for Western blot analysis. Immobilized protein shown is Coomassie-stained and is 20× greater than that shown in binding assay. *I*, *FT*, and *E* designate input, flow through, and elution. Antibodies used for detection are as noted. The presence of Acm1 has no effect on APC/Cdh1 binding.

tion may function then to inhibit APC^{Cdh1} regardless of the phosphorylation status of Cdh1.

Our next question was how CAB complex formation inhibited APC^{Cdh1} activity. Enzymatic activity of APC^{Cdh1} is mediated by the interaction between Cdh1, APC, and the substrate. Therefore, we hypothesized the complex either blocked APC binding to Cdh1 or blocked Cdh1 binding to Clb2. To determine whether Acm1 and Bmh1 were inhibiting APC^{Cdh1} activity by blocking the APC-Cdh1 interaction or by blocking substrate binding to Cdh1, we performed binding assays between Cdh1-APC and Cdh1-Clb2 with or without the presence of the other complex components (Fig. 3, *D* and *E*). Bacterially expressed GST-Clb2 was incubated with FLAG-Cdh1 from *acm1*Δ cells after Cdh1 was preincubated with varying levels of Acm1 in an *in vitro* binding assay. In the second *in vitro* binding assay, N-terminally tagged FLAG-Cdh1 was purified from WT and *acm1*Δ cells and incubated with APC via Apc1-TAP. Although, no difference in Cdh1 binding to the APC could be seen in the presence or absence of Acm1 (Fig. 3*E*, compare *lanes 13* and *17*), Cdh1 binding to Clb2 decreased in a dose-dependent manner with increasing levels of Acm1 (Fig. 3*D*, *lanes 5–8*). This observation suggests Acm1 disrupts the interaction between the substrate and Cdh1, which may explain how CAB complex formation inhibits APC^{Cdh1} activity.

Formation of the Complex Is Phosphorylation-dependent—Current data support the important role Cdh1 phosphorylation plays in the inactivation of APC^{Cdh1} activity. Therefore, we hypothesized phosphorylation had some involvement with the interaction between Cdh1, Acm1, and Bmh1. To determine what role phosphorylation plays in the complex, we employed an *in vitro* binding assay between Bmh1 and Cdh1 with and without phosphatase treatment of Cdh1 (Fig. 4*A*). Cdh1-FLAG was purified from WT cells, treated with λ-phosphatase, and incubated with immobilized GST-Bmh1 in an *in vitro* binding assay. Cdh1-FLAG treated with phosphatase buffer but without λ-phosphatase was used as a control. A shift in Cdh1 due to dephosphorylation can be seen when run on a 7% Tris acetate gel (Fig. 4*A*, *lower panel*). Upon phosphatase treatment, Cdh1 no longer interacted with Bmh1 (compare *lanes 6* and *9*). However, because Cdh1 was purified from WT cells, Acm1 is present. As shown previously, Acm1 is needed for the Cdh1-Bmh1 interaction. Acm1 contains five copies of the consensus CDK recognition sequence ((S/T)P*X*(K/R)), and we have observed that Acm1 is heavily phosphorylated in normal Cdh1 purifications from asynchronous cells. From this assay we could not conclude whether the dephosphorylation of Cdh1, Acm1, or both resulted in the diminished binding of Cdh1 with Bmh1. To determine whether phosphorylation of Cdh1 or Acm1 contribute to formation of the complex, we purified N-terminally tagged FLAG-Cdh1 from *acm1*Δ cells and N-terminally tagged HA-Acm1 from *cdh1*Δ cells and treated each with phosphatase in an *in vitro* binding assay. Surprisingly, we found that phosphorylation is not needed for the interaction between Cdh1 and Acm1, and the addition of Bmh1 had little effect (Fig. 4*B*). To determine whether Acm1 phosphorylation affects Bmh1 binding, an *in vitro* binding assay was employed with immobilized GST-Bmh1 incubated with phosphatase-treated or untreated FLAG-Acm1 from *cdh1*Δ cells. Dephosphorylation of Acm1 abolished binding to Bmh1 indicating that phosphorylation of Acm1 but not Cdh1 is critical for the CAB complex formation (Fig. 4*C*, *lanes 4* and *8*).

Phosphorylated Cdh1 Binds To and Activates the APC in Vitro and Is Inhibited by Complex Formation—Knowing Acm1 has the ability to rescue lethality caused by expressing constitutively active Cdh1 with 9 of the 11 CDK sites mutated to alanine, we hypothesized partially phosphorylated Cdh1 could still bind and activate the APC. First, we investigated whether *in vivo* phosphorylated Cdh1 could still bind to the APC in the absence of the complex. To test this notion, we bound Cdh1 to the APC in an *in vitro* binding assay and then used phosphatase treatment on the complex to illustrate that all bound Cdh1 to the APC is still partially phosphorylated. N-terminally tagged FLAG-Cdh1 was purified from asynchronous *acm1*Δ cells and incubated with yeast-purified APC via Apc1-TAP for 30 min at 4 °C. After incubation with Cdh1, the immobilized APC/bound Cdh1 was washed extensively and divided into two columns, and one column was treated with λ-phosphatase. After treatment, samples were separated by SDS-PAGE and analyzed by Western blot. We found all Cdh1 bound to the APC was phosphorylated. Samples were run on a 6% Tris-glycine gel to differentiate phosphorylated from dephosphorylated Cdh1. A clear mobility shift due to dephosphorylation of bound Cdh1 can be seen from untreated and phosphatase-treated samples (Fig. 5*A*). We believe the Cdh1 bound to the APC is only partially phosphorylated due to the existing evidence that fully phosphorylated Cdh1 cannot bind or activate the APC (16, 18). This suggests partial *in vivo* phosphorylation of Cdh1 in the absence of the complex does not fully inhibit the interaction between Cdh1 and the APC.

Because partial phosphorylation of Cdh1 did not disrupt the interaction with the APC, we investigated whether the phosphorylated co-activator could activate the APC. From the previous experiments, we knew Cdh1 phosphorylation was not a prerequisite for the interaction with Acm1. So, we also examined whether the addition of Acm1 and Bmh1 could attenuate Clb2 ubiquitination using phosphorylated or nonphosphorylated Cdh1. To test this, we purified Cdh1 from *acm1*Δ cells, divided the sample for dephosphorylation by phosphatase, and used phosphorylated and dephosphorylated Cdh1 in the *in vitro* ubiquitination assay (Fig. 5*B*). Cdh1 was washed extensively to remove phosphatase prior to adding Cdh1 to the assay. We added equivalent amounts of Cdh1 to both sets of reactions. Both partially phosphorylated and dephosphorylated Cdh1 were sufficient for APC-mediated ubiquitin ligase activity (*lanes 3* and *8*). Upon the addition of Acm1 and Bmh1, a decrease in Clb2 ubiquitination can be seen using λ-phosphatase treated and untreated Cdh1 (*lanes 4*, *5*, *9*, and *10*). Although Cdh1 phosphorylation is not necessary for Acm1 binding and inhibition, Acm1 and Bmh1 may exist as an early inhibition mechanism of partially phosphorylated Cdh1 before the cyclin-dependent kinase can fully phosphorylate Cdh1 for inactivation. Acm1 and Bmh1 may exist as an overlapping inhibitory mechanism regardless of the phosphorylation status of Cdh1 to ensure proper APC inactivation at the G_1/S phase transition and during S phase.

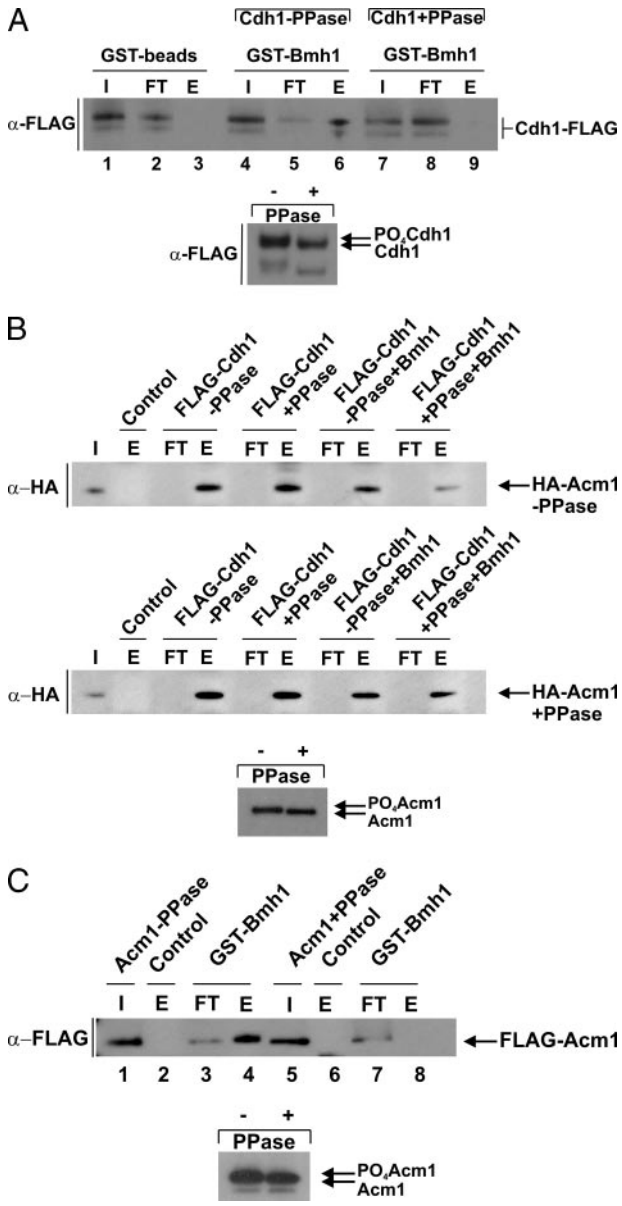

FIGURE 4. **Phosphorylation is involved in the assembly of the CAB complex.** *A*, an *in vitro* binding assay was used with C-terminally tagged Cdh1-FLAG purified from WT cells (YKA154) and treated with λ-phosphatase. Phosphatase-treated and untreated Cdh1 was incubated with bacterially expressed GST-Bmh1 immobilized on GSH affinity beads for 2 h at 4 °C. Once Cdh1 was bound, columns were washed extensively with high salt APC-C buffer and eluted with 1× LDS sample buffer. *I*, *FT*, and *E* designate input, flow through, and elution. Phosphatase treatment of Cdh1 abolished Bmh1 binding. *B*, an *in vitro* binding assay was used with N-terminally tagged FLAG-Cdh1 and HA-Acm1 treated with λ-phosphatase. FLAG-Cdh1 was purified from 2 liters of asynchronous yeast cultures grown to $A_{600} = 1$ and incubated with 100 μl of washed α-FLAG resin. Resin was divided, and one half was treated with phosphatase. Bound FLAG-Cdh1 was used as bait. HA-Acm1 was purified from 2 liters of asynchronous cells grown to $A_{600} = 1$ and incubated with 50 μl of washed α-HA resin. One half was treated with phosphatase and eluted 2× in 50 μl of low salt APC-C buffer with HA peptide at 100 μg/ml final concentration. 1/20 of HA-Acm1 elution was used in each assay and allowed to incubate for 1.5 h at 4 C. Once bound, the proteins were washed extensively with high salt APC-C buffer. 40% of each reaction was loaded on the gel. A shift in dephosphorylation of Acm1 can be seen in the *lower panel*. Empty α-FLAG beads were used as the control. Phosphorylation of Acm1 or Cdh1 has no effect on their interaction with one another. *C*, an *in vitro* binding assay was used with N-terminally tagged and bacterially expressed GST-Bmh1. GST and GST-Bmh1 were prepared as described previously. N-terminally tagged FLAG-Acm1 was purified from 2 liters of asynchronous yeast cultures grown to $A_{600} = 1$, purified using 50 μl of α-FLAG resin, and one half treated with λ-phosphatase. FLAG-Acm1 was eluted with FLAG peptide (250 μg, final concentration) in 50 μl of low salt APC-C buffer. Bound GST-Bmh1 (10 μl of GSH beads) was used as bait and incubated for 30 min at 4 °C with either phosphatase-treated or untreated FLAG-Acm1. 1/25 of FLAG-Acm1 elution was used in each assay. Bound proteins were washed extensively with high salt APC-C buffer and eluted in LDS sample buffer. 20% from each assay was loaded on the gel. Nonphosphorylated Acm1 does not interact with Bmh1.

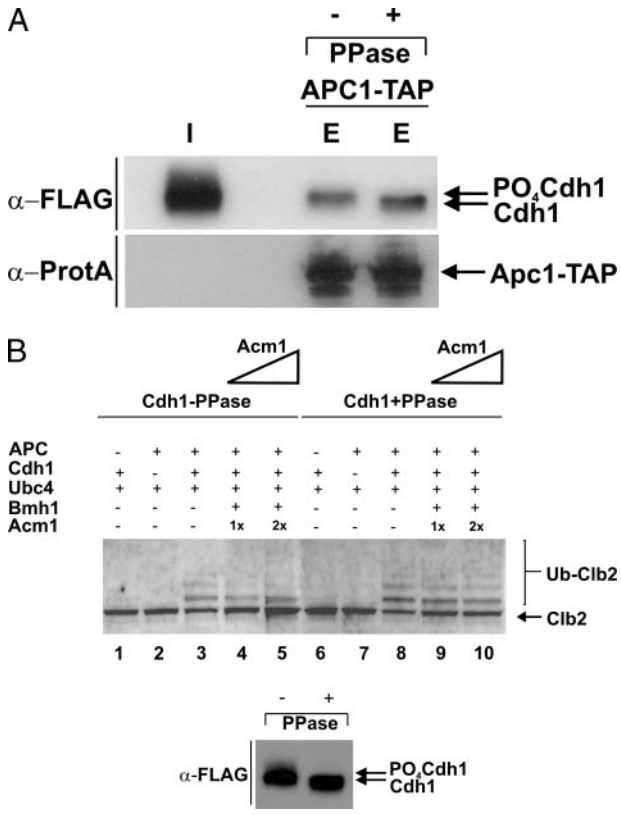

FIGURE 5. **Phosphorylated Cdh1 is inhibited by complex formation.** *A*, N-terminally tagged FLAG-Cdh1 was purified from *acm1*Δ cells and incubated with APC via Apc1-TAP immobilized on calmodulin beads. After incubation with Cdh1, the immobilized APC/bound Cdh1 was washed extensively and divided into two columns, and one was treated with λ-phosphatase. After treatment, samples were separated on a 6% Tris-glycine gel to differentiate phosphorylated Cdh1 from unphosphorylated Cdh1 and analyzed by Western blot. Antibodies used for detection are as noted. Partially phosphorylated Cdh1 can bind the APC. *B*, Ubc4 and Clb2 used in this assay were produced by *in vitro* transcription/translation. N-terminally tagged FLAG-Cdh1 was purified from 2 liters of asynchronous yeast cultures and incubated with 50 μl of α-FLAG resin. The beads were divided and washed extensively with APC-C buffer, and one half was treated with λ-phosphatase for dephosphorylation. 1/50 of the FLAG-Cdh1 elution was used in each assay. Acm1 was purified from 2 liters of asynchronous cells grown to $A_{600} = 1$ using 100 μl of washed calmodulin resin. Resin was washed extensively with APC-C buffer containing 1 mM $CaCl_2$ and eluted in 2.5 mM EDTA buffer. 15 mM $MgCl_2$ was added to the eluted Acm1 before use in the ubiquitination assay. The *triangle* represents increasing levels of Acm1. 1× Acm1 is the equivalent of 2 μl or 1/50 from the EDTA elution. Each reaction contains components designated by "+" and "−." Components of the assay were mixed and incubated as described previously. Ubiquitination of Clb2 can be seen as bands of decreasing gel mobility. Partially phosphorylated Cdh1 activates the APC, and Acm1 and Bmh1 inhibit phosphorylated and nonphosphorylated Cdh1.

DISCUSSION

Previous work has shown the phosphorylation of Cdh1 occurs at the end of G_1 phase by the cyclin-dependent kinase Cdc28 and has demonstrated that phosphorylation is responsible for the inactivation of the APC^{Cdh1} complex (16, 18, 31). Non-phosphorylatable Cdh1 mutants constitutively activate

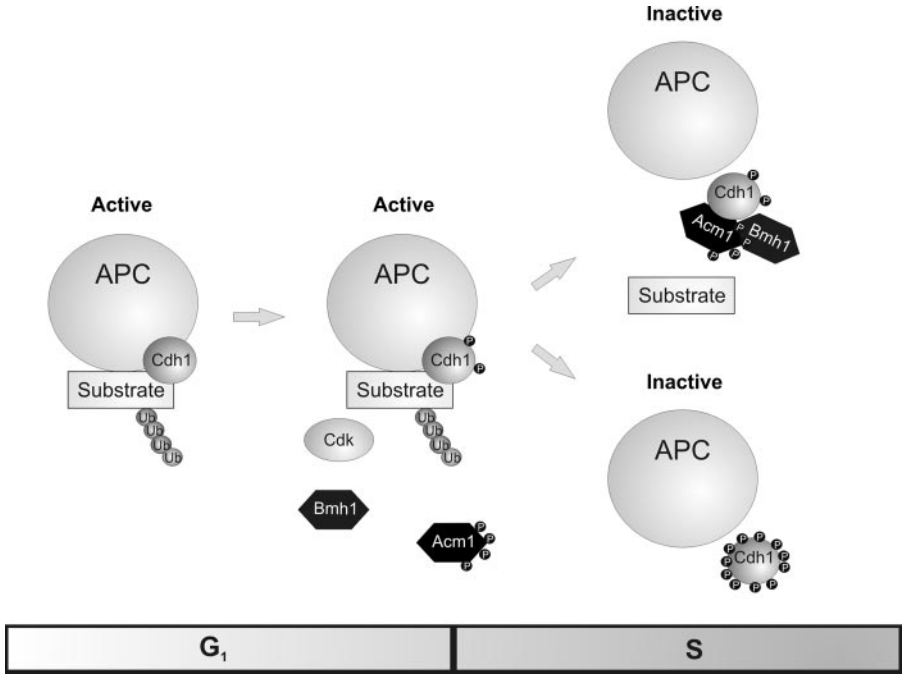

FIGURE 6. **Model of APC^{Cdh1} inactivation.** APC^{Cdh1} is active throughout G_1 phase until Cdh1 is phosphorylated by the cyclin-dependent kinase Cdc28 at the G_1/S phase transition. Additionally, a complex forms with the co-activator consisting of Acm1 and Bmh1, which inhibits the APC at the end of G_1. The CAB complex dissociates the substrate from Cdh1 and acts independently of Cdh1 phosphorylation for the timely inactivation of the APC.

the APC prohibiting cells from accumulating Clb2 and Clb3 (16). Additionally, *in vitro* phosphorylation of recombinant Cdh1 is convincingly sufficient for inactivation of the APC (18). There is no question phosphorylation of Cdh1 plays an important role in APC inactivation. Because the cell cycle follows a very strict schedule, proper and timely inactivation of the APC must occur. Because Cdh1 has been shown to bind substrates independent of the APC (9, 14, 32), one reason for the strict regulation of Cdh1 may be to prevent the co-activator from interfering with substrates during parts of the cell cycle where it is not needed. It is logical then that cells have overlapping inactivation mechanisms to be sure certain cellular processes are disabled. The possibility remains for novel proteins to exist for APC^{Cdh1} inactivation. Here, we have identified an additional mechanism that acts independently of Cdh1 phosphorylation to efficiently inhibit Cdh1 from activating the APC. The mechanism is composed of complex formation of Acm1 and Bmh1 with Cdh1 (CAB complex) and functions to inhibit APC^{Cdh1} activity by blocking substrate binding to the co-activator. The CAB complex is the first APC^{Cdh1} inhibitor identified in yeast. Acm1 is the critical component involved in complex formation and is cell cycle-regulated. Hence, the Cdh1-Bmh1 interaction is confined to the cell cycle interval during which Acm1 is expressed.

We identified Acm1, Bmh1, and Bmh2 as Cdh1-interacting proteins using Cdh1 immunoprecipitations. *BMH1* and *BMH2* appear to be functionally redundant, and although deletion of both is lethal in most strains, cells remain viable with the deletion of only one of the genes (33, 34). Bmh1 and Bmh2 are members of the 14-3-3 class of proteins whose roles include signal transduction, checkpoint control, apoptotic, and nutrient-sensing pathways, as well as subcellular localization of binding partners (26, 35). 14-3-3 proteins have been defined as phosphoserine-binding proteins with ligands typically containing two different binding motifs, RS*X*pS*X*P or R*X*(Y/F)*X*pS*X*P, although variations of these motifs exist (25, 36). The third member of the complex, which forms with Cdh1, is a previously uncharacterized protein that has been named Acm1. Acm1 has been shown to interact with Bmh2 via yeast two-hybrid assays (37). Acm1 has been identified as a potential Cdc28 substrate and contains five potential CDK phosphorylation sites that match the consensus recognition sequence (S/T)P*X*(K/R) (24). Interestingly, the gene expression pattern of Acm1 is similar to that of Clb5, whose accumulation is necessary for Cdh1 phosphorylation and S phase entry. Although we have not seen any significant sequence homology of Acm1 in mammalian species, the possibility remains an analog exists or that 14-3-3 proteins do not need an adaptor protein like Acm1 in higher eukaryotes.

Inhibition of Cdh1 must occur in a timely manner for S phase to proceed. The presence of Acm1 is therefore necessary at the specific time when APC^{Cdh1} needs to be inactivated. Indeed, protein levels of Acm1 are highest in S phase coinciding with the phosphorylation of Cdh1 by Clb5-Cdc28. Deletion of Acm1 causes a perturbation in the cell cycle leading to an increased population of cells in S phase. Currently, we cannot decipher whether the increased S phase population is a result of early entry into S phase or slower progression through S phase. Additionally, we have observed slightly lower Clb2 levels in *acm1*Δ cells after a G_1 arrest and release suggesting an increased APC^{Cdh1} activity in the absence of Acm1. The lower levels of Clb2 occurred in early time points under certain growth conditions. However, Clb2 levels return to a comparable level at later time points in the same experiment that we attribute to effective inhibition of APC^{Cdh1} by phosphorylation. The cell cycle perturbation may not be explained by differing Clb2 levels, but a longer S phase as a result of lower CDK activity could occur (38). However, our data can only suggest Acm1 has some regulatory role during S phase. Further experiments are needed to determine whether levels of S phase cyclins differ in *acm1*Δ cells.

Phosphorylation of Cdh1 occurs in S phase, and complete phosphorylation disrupts the binding interface between Cdh1 and the APC. We have shown *in vivo* phosphorylated Cdh1 binds to and activates the APC, although this result can be explained if Cdh1 is only partially phosphorylated. We have demonstrated Acm1 and Bmh1 have a direct inhibitory effect on APC^{Cdh1} activity *in vitro* and *in vivo* and that inhibition by

Acm1 and Bmh1 can occur when Cdh1 is phosphorylated or dephosphorylated. Although the phosphorylation status of Cdh1 does not affect CAB complex formation, phosphorylation of Acm1 plays a critical role in the assembly of the ternary complex with Bmh1 and Cdh1. We have identified several phosphopeptides within Acm1 from Cdh1 immunoprecipitations. This is not surprising, because Acm1 was originally identified as a potential Cdc28 substrate (24). Dephosphorylation of purified Acm1 has no effect on Cdh1 binding but does abolish Bmh1 binding. Phosphorylation of Acm1 and Cdh1 may occur in parallel by the same kinase to mediate complex formation. We have also observed a slight shift in the gel mobility of Acm1 before it is degraded. The gel shift could be a result of dephosphorylation, which may play a role in Acm1 stability. Because Bmh1 binds only phosphorylated Acm1 and has no inhibitory role by itself, it may serve to protect Acm1 from early degradation. We are currently determining whether phosphorylation of Acm1 is required for APC^{Cdh1} inhibition and if it is involved in Acm1 proteolysis.

We have not thoroughly explored the possibility of Acm1 and Bmh1 as novel APC substrates. Protein levels of Acm1 drop sharply after S phase when the APC is reactivated. Although *ACM1* mRNA expression drops after S phase, the residual Acm1 left over may need to be removed to prevent premature inhibition of APC^{Cdh1}. Proteasomal degradation mediated by APC^{Cdc20} would make sense to remove Acm1 until it is needed for APC^{Cdh1} inactivation. The destruction box (D box) and the KEN box have been identified as degradation motifs in APC substrates (14), whereas Acm1 contains two potential D boxes (RTIL at amino acid 8 and RIAL at amino acid 119) and a potential KEN box (KENLS at amino acid 98). Unlike APC substrates, Bmh1 levels are abundant throughout the cell cycle, and its levels do not fluctuate. Additionally, Bmh1 does not interact with Cdh1 without Acm1 present. For these reasons it is unlikely Bmh1 is an APC substrate. However, further investigation is needed to determine the ultimate fate of these proteins.

Interestingly, there appear to be some similarities between the APC^{Cdh1} inhibitor, Emi1, in higher eukaryotes and the CAB complex in yeast. Both inhibit APC^{Cdh1} activity *in vitro* and directly bind Cdh1 (39). Emi1 and Acm1 are both present during S phase and are degraded during M phase (40). In a recent report, a model system has been proposed where Emi1 acts as a pseudosubstrate inhibitor of APC^{Cdh1} and competes with other D box-containing substrates for Cdh1 binding (41). Although we have demonstrated Acm1 and Bmh1 block binding between Cdh1 and the APC substrate Clb2, we have not yet determined which regions in Acm1 are involved in Cdh1 binding or if Acm1 mimics an APC substrate. We have observed an interaction between Acm1 and C-terminal fragments of Cdh1 in gel-filtration experiments. The C terminus of Cdh1 houses a WD-40 domain shown to be the site for substrate binding (9). We can only speculate that, like Emi1, Acm1 could also mimic Cdh1 substrates to inhibit APC^{Cdh1}. On the other hand, there is no sequence similarity between Emi1 and Acm1 outside of their D box and KEN box regions. Thus, it is not clear whether these Cdh1-associated proteins share the same inhibitory role between species. We are currently exploring the mechanism of inhibition of the CAB complex in greater detail.

Cdh1 has been shown to relocalize from the nucleus to the cytoplasm removing it from the vicinity of the APC after phosphorylation presumably as a redundant inhibitory mechanism (42). 14-3-3 proteins are involved in the relocalization of the cell cycle regulatory protein Cdc25, so feasibly Acm1 and Bmh1 could play a similar role with Cdh1 (43, 44). However, the possibility of Acm1 and Bmh1 affecting Cdh1 localization has not been thoroughly investigated.

Conceivably, additional mechanisms exist for APC inactivation through other protein interactions (45, 46). Our evidence suggests that the complex formation serves as an overlapping mechanism in addition to the phosphorylation message to efficiently inhibit APC^{Cdh1} activity prior to entering mitosis (Fig. 6). However, additional studies are required to further elucidate the biological significance and mechanism by which CAB complex formation inhibits APC^{Cdh1}.

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